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Signed Andrew Gurney

Dated 30 October 2003

[redacted]2 E753785-1 D02973
P01/7700 0.00-0223193.4

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Request for grant of a patent

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7 OCT 2002

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1. Your reference — P101117GB

2. Patent application number *(The Patent Office will fill in this part)* — 0223193.4

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Ludwig Institute for Cancer Research
Postfach 8024
ZURICH
Switzerland

Patents ADP number *(if you know it)* 57715500 2.

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — Polypeptide

5. Name of your agent *(if you have one)*

Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

31 St Saviourgate
YORK
YO1 8NQ

Patents ADP number *(if you know it)* ~~14571001~~ 7914237002.

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

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a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

yes

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Continuation sheets of this form

Description	33
Claim(s)	6
Abstract	1
Drawing(s)	13

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Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Harrison Goddard Foot Date 7 October 2002

12. Name and daytime telephone number of person to contact in the United Kingdom — Robert C Docherty — 01904 732120

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POLYPEPTIDE

The invention relates to a polypeptide, or part thereof, which inhibits the apoptotic activity of the tumour suppressor protein p53 and including screening methods to identify agents which interfere with the activity of said polypeptide.

Apoptosis, or programmed cell death, is a process by which multi-cellular organisms regulate cell number and differentiation. The process is regulated by factors which either induce or prevent apoptosis. Inducers of apoptosis include Bcl-2 family members, caspase family members and their associated factors Apaf-1 and Fadd. Caspases are synthesised as proenzymes which become activated after proteolytic cleavage. The active caspase then induces many of the morphological and biochemical changes associated with apoptosis. Mitochondria play a pivotal role in the activation process through the release of pro-apoptotic factors such as cytochrome c, AIF and Diablo. The release from mitochondria is controlled by the Bcl-2 family of proteins; (e.g. Bcl-2 and Bcl-xl inhibit release; Bax and Bak induce release). WO9953051 discloses a cytokine dependent protein p21 which has pro-apoptotic activity. p21 is expressed in a cytokine dependent manner in myeloid/erthyroid cells. These cells are dependent on IL-3 for growth and in the absence of IL-3 the translation of p21 is induced resulting in apoptosis and cell death. p21 is a cytoplasmic protein which translocates to the outer mitochondrial membrane to induce pro-apoptotic activities.

Tumour suppressor proteins also have pro-apoptotic activities.

Tumour suppressor genes encode proteins which function to inhibit cell growth or division and are therefore important with respect to maintaining proliferation, growth and differentiation of normal cells. Mutations in tumour suppressor genes result in abnormal cell-cycle progression whereby the normal cell-cycle check points which arrest the cell-cycle, when, for example, DNA is damaged, are ignored and damaged cells divide uncontrollably. The products of tumour suppressor genes function in all

parts of the cell (e.g. cell surface, cytoplasm, nucleus) to prevent the passage of damaged cells through the cell- cycle (i.e. G1, S, G2, M and cytokinesis).

Arguably the tumour suppressor gene which has been the subject of the most intense research is p53. p53 encodes a protein which functions as a transcription factor and is a key regulator of the cell division cycle. It was discovered in 1978 as a protein shown to bind with affinity to the SV40 large T antigen. The p53 gene encodes a 393 amino acid polypeptide with a molecular weight of 53kDa. Genes regulated by the transcriptional activity of p53 contain a p53 recognition sequence in their 5' regions. These genes are activated when the cellular levels of p53 are elevated due to, for example DNA damage. Examples of genes which respond to p53 include, mdm2, Bax and PIG-3. Bax and PIG-3 are involved in one of the most important functions of p53, the induction of apoptosis.

In our co-pending application WO02/12325 we disclose a family of proteins, referred to as ASPP, as specific activators of p53 and revealed a mechanism by which wild type p53 is tolerated in tumours, such as human breast carcinomas. We also disclose an inhibitor of ASPP family members referred to as iASPP. iASPP is an oncogene and is the most conserved member of the ASPP family. iASPP is the only ASPP-like protein found in *C. elegans*. Similar to human iASPP, the *C. elegans* homologue functions as a key inhibitor of p53. These findings indicate that regulation of p53 function by members of the ASPP family has been evolutionarily conserved across phyla.

The *C. elegans* iASPP is capable of substituting for human iASPP in all of the assays performed in human cells. Moreover, reciprocal substitution studies reveal that the apoptotic function of *C. elegans* p53 is enhanced or inhibited by human ASPP and iASPP, respectively. Using RNAi we further demonstrate that iASPP is a key inhibitor of p53 mediated apoptosis in C. elegans. All of these observations show that the regulation of p53 by ASPP family members is evolutionarily conserved. Control of p53 activity plays a pivotal role in development and tumourigenesis. Hence,

inhibiting the oncogenic function of iASPP could provide an important new strategy for treating tumours expressing wild type p53. Sequence comparision between *C.elegans* and human iASPP reveals a conserved domain between the nematode and human sequence which likely explains the functional conservation between the proteins.

According to an aspect of the invention there is provided an isolated nucleic acid molecule which encodes a polypeptide, or sequence variant thereof, wherein said polypeptide is a fragment of the polypeptide sequence represented in Figure 1a or 1b, which fragment is selected from the group consisting of:

i) a polypeptide fragment consisting of amino acid residues from about residue 128-224 of the amino acid sequence presented in Figure 1a or 1b;

ii) a polypeptide fragment consisting of amino acid residues from about 128-244 of the amino acid sequence presented in Figure 1a or 1b wherein said sequence has been modified by addition, deletion or substitution of at least one amino acid residue; and

iii) a polypeptide as defined in (i) and (ii) wherein said polypeptide substantially retains the biological activity of the polypeptide represented in Figure 1a or 1b.

In a preferred embodiment of the invention said nucleic acid molecule encodes a fragment consisting of amino acid residues from about 128-224 of the sequence represented in Figure 1a. Preferably said nucleic acid molecule is isolated from a human.

In an alternative preferred embodiment of the invention said nucleic acid molecule encodes a fragment consisting of amino acid residues from about 128-224 of the sequence represented in Figure 1b. Preferably said nucleic acid molecule is isolated from a nematode. Preferably said nematode is of the genus *Caenorhabditis* spp.

In a preferred embodiment of the invention said nucleic acid molecule encodes a polypeptide, or sequence variant thereof, which polypeptide inhibits the activity of a polypeptide represented by the amino acid sequence represented in Figure 1a or 1b.

In a preferred embodiment of the invention said nucleic acid molecule is a cDNA.

In an alternative preferred embodiment of the invention said nucleic acid molecule is genomic DNA.

According to a further aspect of the invention there is provided a polypeptide fragment or sequence variant thereof, encoded by a nucleic acid molecule according to the invention.

It will be apparent that fragments which are sequence variants may retain the biological activity of the full length polypeptide or alternatively have antagonistic activity by competing for binding sites in p53. In general, the specificity of polypeptides according to the invention with respect to binding to p53 is shown by binding equilibrium constants. Polypeptides which are capable of selectively binding p53 preferably have binding equilibrium constants of at least about 10^7 M^{-1}, more preferably at least about 10^8 M^{-1}, and most preferably at least about 10^9 M^{-1}.

A sequence variant , i.e. a fragment polypeptide and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and asparatic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalaine, tyrosine and tryptophan.

A functionally equivalent polypeptide according to the invention is a variant wherein one in which one or more amino acid residues are substituted with conserved or non-conserved amino acid residues, or one in which one or more amino acid residues includes a substituent group. Conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among aromatic residues Phe and Tyr.

In addition, the invention features polypeptide sequences having at least 75% identity with the polypeptide sequences as hereindisclosed, or fragments and functionally equivalent polypeptides thereof. In one embodiment, the polypeptides have at least 85% identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the amino acid sequences illustrated herein.

As mentioned above, the invention also provides, in certain embodiments, "dominant negative" polypeptides derived from the polypeptides hereindisclosed. A dominant negative polypeptide is an inactive variant of a protein, which, by interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or competes with the active protein, thereby reducing the effect of the active protein. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand. Likewise, a dominant negative catalytically-inactive kinase which interacts normally with target proteins but does not phosphorylate the target proteins can reduce phosphorylation of the target proteins in response to a cellular signal. Similarly, a dominant negative transcription factor which binds to another transcription factor or to a promoter site in the control region of a gene but does not increase gene transcription can reduce the effect of a

normal transcription factor by occupying promoter binding sites without increasing transcription.

The end result of the expression of a dominant negative polypeptide in a cell is a reduction in function of active proteins. One of ordinary skill in the art can assess the potential for a dominant negative variant of a protein, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of iASPP polypeptides, one of ordinary skill in the art can modify the sequence of iASPP polypeptides by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in a selected activity (e.g., p53 binding, modulation of apoptosis) and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a protein will be apparent to one of ordinary skill in the art.

According to a further aspect of the invention there is provided a vector comprising a nucleic acid according to the invention.

In a further preferred method of the invention said vector is an expression vector conventionally adapted for gene expression.

Typically said adaptation includes, by example and not by way of limitation, the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive.

Promoter is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only, and not by way of limitation. Enhancer

elements are cis acting nucleic acid sequences often found 5' to the transcription initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences and is therefore position independent). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to trans acting transcription factors (polypeptides) which have been shown to bind specifically to enhancer elements. The binding/activity of transcription factors (please see Eukaryotic Transcription Factors, by David S Latchman, Academic Press Ltd, San Diego) is responsive to a number of environmental cues which include, by example and not by way of limitation, intermediary metabolites (eg glucose, lipids), environmental effectors (eg light, heat,).

Promoter elements also include so called TATA box and RNA polymerase initiation selection (RIS) sequences which function to select a site of transcription initiation. These sequences also bind polypeptides which function, inter alia, to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers and autonomous replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors which are maintained autonomously are referred to as episomal vectors. Episomal vectors are desirable since these molecules can incorporate large DNA fragments (30-50kb DNA). Episomal vectors of this type are described in WO98/07876.

Adaptations which facilitate the expression of vector encoded genes include the provision of transcription termination/polyadenylation sequences. This also includes the provision of internal ribosome entry sites (IRES) which function to maximise expression of vector encoded genes arranged in bicistronic or multi-cistronic expression cassettes.

Expression control sequences also include so-called Locus Control Regions (LCRs). These are regulatory elements which confer position-independent, copy number-dependent expression to linked genes when assayed as transgenic constructs in mice. LCRs include regulatory elements that insulate transgenes from the silencing effects of adjacent heterochromatin, Grosveld et al., Cell (1987), 51: 975-985.

These adaptations are well known in the art. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.(1994).

According to a further aspect of the invention there is provided a cell tranformed or transfected with a nucleic acid molecule or vector according to the invention.

Preferably, said host cells are eukaryotic cells, for example, insect cells such as cells from a species Spodoptera frugiperda using a baculovirus expression system. This expression system is favoured in the instance where post-translational modification of the polypeptide is required. Host cells and cell lines, can be prokaryotic (e.g., E. coli), or eukaryotic (e.g., CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as human, mouse, hamster, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include keratinocytes, peripheral blood leukocytes, fibroblasts, bone marrow stem cells and embryonic stem cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described above, be operably linked to a promoter.

According to a further aspect of the invention the invention there is provided a polypeptide according to the invention for use as a pharmaceutical.

According to a further aspect of the invention there is provided a nucleic acid according to the invention for use as a pharmaceutical.

In a preferred embodiment of the invention said pharmaceutical further comprises a a diluent, carrier or excipient.

When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents, such as chemotherapeutic agents.

The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. When antibodies are used therapeutically, a preferred route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing antibodies are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Sciarra and Cutie, "Aerosols," in Remington's Pharmaceutical Sciences, 18th edition, 1990, pp 1694-1712; incorporated by reference). Those of skill in the art can readily determine the various parameters and conditions for producing antibody aerosols without resort to undue experimentation. When using antisense preparations of the invention, slow intravenous administration is preferred.

The compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a composition that alone, or together with further doses, produces the desired response. In the case of treating a particular disease, such as cancer, the desired response is inhibiting the progression of the disease. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The pharmaceutical compositions used in the foregoing methods preferably are sterile and contain an effective amount of dominant negative iASPP or nucleic acid encoding a dominant negative iASPP, for producing the desired response in a unit of weight or volume suitable for administration to a patient. The response can, for example, be measured by determining the signal transduction inhibited by the dominant negative iASPP-1, composition via a reporter system as described herein, by measuring downstream effects such as gene expression, or by measuring the physiological effects of the iASPP composition, such as regression of a tumor, decrease of disease symptoms, modulation of apoptosis, etc.

The doses of dominant negative iASPP polypeptide or nucleic acid administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

In general, doses of dominant negative iASPP are formulated and administered in doses between 1 ng and 1 mg, and preferably between 10 ng and 100 μg, according to any standard procedure in the art. Where nucleic acids encoding dominant negative iASPP are employed, doses of between 1 ng and 0.1 mg generally will be formulated and administered according to standard procedures. Other protocols for the administration of iASPP compositions will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration (e.g., intra-tumoral) and the like vary from the foregoing. Administration of iASPP compositions to mammals other than humans, e.g. for testing purposes or veterinary therapeutic purposes, is carried out under substantially the same conditions as described above. A subject, as used herein, is a mammal, preferably a human, and including a non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the

invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

iASPP compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into

association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of iASPP polypeptides or nucleic acids, which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

According to a further aspect of the invention there is provided a transgenic non-human animal comprising a nucleic acid according to the invention.

The invention also includes transgenic non-human animals. As used herein, "transgenic non-human animals" includes non-human animals having one or more exogenous nucleic acid molecules incorporated in germ line cells and/or somatic cells. Thus the transgenic animal include "knockout" animals having a homozygous or heterozygous gene disruption by homologous recombination, animals having

episomal or chromosomally incorporated expression vectors, etc. Knockout animals can be prepared by homologous recombination using embryonic stem cells as is well known in the art. The recombination can be facilitated by the cre/lox system or other recombinase systems known to one of ordinary skill in the art. In certain embodiments, the recombinase system itself is expressed conditionally, for example, in certain tissues or cell types, at certain embryonic or post-embryonic developmental stages, inducibly by the addition of a compound which increases or decreases expression, and the like. In general, the conditional expression vectors used in such systems use a variety of promoters which confer the desired gene expression pattern (e.g., temporal or spatial). Conditional promoters also can be operably linked to iASPP family nucleic acid molecules to increase expression of these nucleic acid molecules in a regulated or conditional manner. Trans-acting negative regulators of iASPP activity or expression also can be operably linked to a conditional promoter as described above. Such trans-acting regulators include antisense nucleic acids molecules, nucleic acid molecules which encode dominant negative molecules, ribozyme molecules specific for iASPP nucleic acids, and the like. The transgenic non-human animals are useful in experiments directed toward testing biochemical or physiological effects of diagnostics or therapeutics for conditions characterized by increased or decrease iASPP expression. Other uses will be apparent to one of ordinary skill in the art.

According to a further aspect of the invention there is provided the use of the polypeptide, or fragment thereof, in a screening method for the identification of agents which inhibit the binding of said polypeptide to p53.

According to a further aspect of the invention there is provided a screening method to identify agents which inhibit the binding of a polypeptide or fragment thereof to p53 comprising:

i) forming a preparation comprising

a) a polypeptide according to the invention; and

b) a p53 polypeptide, or a fragment thereof consisting of the binding site(s) for the polypeptide in (a);

ii) providing at least one agent to be tested; and

iii) determining the activity of the agent with respect to the binding of the polypeptide in (a) to the polypeptide in (b).

In a preferred method of the invention said agent is a polypeptide, preferably a peptide.

In preferred embodiment of the invention said peptide comprises an amino acid sequence selected from the group consisting of: DGPEETD; TTLSDG; AEFGDE; or PRNYFG.

In a preferred embodiment of the invention said peptide is at least 6 amino acid residues in length. Preferaby the length of said peptide is selected from the group consisting of: at least 7 amino acid residues; 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues in length. Alternatively the length of said peptide is at least 20 amino acid residues; 30; 40; 50; 60; 70; 80; 90; or 100 amino acid residues in length.

In a further preferred embodiment of the invention said peptide consists of an amino acid sequence consisting of: DGPEETD; TTLSDG; AEFGDE; or PRNYFG.

It will be apparent to one skilled in the art that modification to the amino acid sequence of peptides agents could enhance the binding and/or stability of the peptide with respect to its target sequence. In addition, modification of the peptide may also increase the *in vivo* stability of the peptide thereby reducing the effective amount of peptide necessary to inhibit p53 binding of iASPP. This would advantageously reduce undesirable side effects which may result in vivo. Modifications include, by example and not by way of limitation, acetylation and amidation. Alternatively or preferably, said modification includes the use of modified amino acids in the

production of recombinant or synthetic forms of peptides. It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N^6-acetyllysine, N^6-methyllysine, N^6,N^6-dimethyllysine, N^6,N^6,N^6-trimethyllysine, cyclohexyalanine, D-amino acids, ornithine. Other modifications include amino acids with a C_2, C_3 or C_4 alkyl R group optionally substituted by 1, 2 or 3 substituents selected from halo (eg F, Br, I), hydroxy or C_1-C_4 alkoxy.

It will also be apparent to one skilled in the art that peptides which retain p53 binding activity could be modified by cyclisation. Cyclisation is known in the art, (see Scott *et al* Chem Biol (2001), 8:801-815; Gellerman et al J. Peptide Res (2001), 57: 277-291; Dutta *et al* J. Peptide Res (2000), 8: 398-412; Ngoka and Gross J Amer Soc Mass Spec (1999), 10:360-363.

In a further preferred method of the invention said antagonist is an antibody or antibody binding part. Preferably said antibody is a monoclonal antibody or binding part thereof.

Antibodies, also known as immunoglobulins, are protein molecules which usually have specificity for foreign molecules (antigens). Immunoglobulins (Ig) are a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light (L) (low molecular weight) chain (κ or λ), and one pair of heavy (H) chains (γ, α, μ, δ and ε), all four linked together by disulphide bonds. Both H and L chains have regions that contribute to the binding of antigen and that are highly variable from one Ig molecule to another. In addition, H and L chains contain regions that are non-variable or constant.

The L chains consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the "constant" (C) region. The amino terminal domain varies from L chain to L chain and contributes to

the binding site of the antibody. Because of its variability, it is referred to as the "variable" (V) region.

The H chains of Ig molecules are of several classes, α, μ, σ, α, and γ (of which there are several sub-classes). An assembled Ig molecule consisting of one or more units of two identical H and L chains, derives its name from the H chain that it possesses. Thus, there are five Ig isotypes: IgA, IgM, IgD, IgE and IgG (with four sub-classes based on the differences in the 'constant' regions of the H chains, i.e., IgG1, IgG2, IgG3 and IgG4). Further detail regarding antibody structure and their various functions can be found in, Using Antibodies: A laboratory manual, Cold Spring Harbour Laboratory Press.

In a preferred embodiment of the invention said fragment is a Fab fragment.

In a further preferred embodiment of the invention said antibody is selected from the group consisting of: $F(ab')_2$, Fab, Fv and Fd fragments; and antibodies comprising CDR3 regions.

A modified antibody, or variant antibody, and reference antibody, may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and asparatic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants which show enhanced biological activity.

Preferably said antibody is a humanised or chimeric antibody.

A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody.

A humanised antibody is produced by recombinant methods to combine the complementarity determining regions (CDRs) of an antibody with both the constant (C) regions and the framework regions from the variable (V) regions of a human antibody.

Chimeric antibodies are recombinant antibodies in which all of the V-regions of a mouse or rat antibody are combined with human antibody C-regions. Humanised antibodies are recombinant hybrid antibodies which fuse the complimentarity determining regions from a rodent antibody V-region with the framework regions from the human antibody V-regions. The C-regions from the human antibody are also used. The complimentarity determining regions (CDRs) are the regions within the N-terminal domain of both the heavy and light chain of the antibody to where the majority of the variation of the V-region is restricted. These regions form loops at the surface of the antibody molecule. These loops provide the binding surface between the antibody and antigen.

Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are therefore thought to be less immunogenic than chimeric antibodies.

According to a further aspect of the invention there is provided an isolated nucleic acid molecule wherein said molecule is isolated from a nematode worm which nucleic acid molecule hybridises a nucleic acid sequence as represented by Fig 1b.

In a preferred embodiment of the invention said nucleic acid molecule hybridises under stringent hybridisation conditions. Preferably said nematode worm is of the genus *Caenorhabditis spp.*

According to a further aspect of the invention there is provided an isolated polypeptide comprising the amino acid as represented in Figure 1b which polyeptide is modified by addition, deletion or substitution of at least one amino acid residue.

According to a further aspect of the invention there is provided a method of treatment of an animal comprising administering an effective amount of a polypeptide or nucleic acid or vector according to the invention wherein said effective amount induces the apopoptic activity of p53.

In a preferred method of the invention said treatment is of cancer.

An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1a is the nucleic acid sequence of human iASPP; Figure 1b is the C.elegans nucleic acid sequence of iASPP;

Figure 2a is the amino acid sequence of human iASPP; Figure 2b is the C.elegans amino acid sequence of iASPP;

Figure 3 illustrates that FITC labelled peptide (3a)DGPEETD and (3b) TTLSDG can penetrate cells;

Figure 4 illustrates the stimulation of the Bax promoter by p53 after incubation with various peptides, in particular DGPEETD;

Figure 5 illustrates the stimulation of p53 transactivation in a human tumour cell line U2SO after UV damage of DNA in the presence of peptide DGPEETD;

Figure 6 illustrates various experiments showing the interaction of C.elegans iASPP and p53;

Figure 7 illustrates a homology comparison between C.elegans iASPP and human iASPP; and

Figure 8 illustrates further experiments showing the interaction of C.elegans Iaspp and p53; and

Figure 9 illustrates the effect of RNAi on C.elegans iASPP expression.

Materials and Methods

Cell culture, antibodies and plasmids

Saos-2, MCF-7, and U2OS cells were grown in DMEM supplemented with 10% FCS, 100 IU/ml penicillin-streptomycin and 2 mM glutamine. Anti-p53 antibodies DO-1 and DO-13 are monoclonal antibodies while CM-1 is a rabbit polyclonal antibody specific to p53. The V5 and 9E10 epitopes are recognised by the mouse monoclonal antibodies V5 and 9E10 respectively. The mouse monoclonal PC-10 is specific to the PCNA protein. CD20Leu is an FITC conjugated monoclonal antibody specific for the cell surface marker CD20 (Becton Dickinson). The mouse and rabbit antibodies to ASPP1 and ASPP2 were described previously[1]. Mouse and rabbit antibody to iASPP (peptide RLQPALPPEAQSVPELEE) was produced as described by Harlow and Lane [13]. All expression plasmids used in this study were driven by

the CMV immediate early promoter. ASPP1, iASPP and Ce-iASPP are tagged with V5 epitope while Ce-p53 is tagged with 9E10 epitope.

DNA transfection

The transfection mix included the DNA of interest in 1X HBS buffer (280 mM NaCl, 10 mM KCl, 1.4 mM $Na_2HPO_4.2H_2O$, 12 mM Glucose, 39 mM HEPES, adjusted to pH 6.9-7.3) precipitated with 2.5 M $CaCl_2$. The transfection mix was added dropwise to the cells and washed off after 6 hours with DMEM. 16-24 hours following the wash, cells were lysed either in Reporter lysis buffer (Promega) for Luciferase assays and western blots or in NP40 lysis buffer for western or immunoprecipitation procedures.

Transactivation assays

For transcriptional assay, 5 x 10^5 Saos-2 cells were plated 24 hr prior to transfection in 6 cm dishes. Various combinations of plasmid DNA were transfected using the following amounts. All transfection assays contain 1μg of reporter plasmid. 50 ng of wild type human p53, 100 ng of plasmid expressing C. elegans p53, 4μg of ASPP2, or 8μg of ASPP1, 5μg of human iASPP or 7.5μg of Ce-iASPP were used as indicated. After transfection, the cells were lysed in Reporter Lysis Buffer (Promega, WI, USA) 16-24 hr post-wash and assayed using the Luciferase Assay kit (Promega, WI, USA). The fold activation of a particular reporter was determined by the activity of the transfected plasmid above the activity of vector alone. The fold increase of p53 transactivation activity by ASPP was obtained by the activity p53 in combination with ASPP divided by the activity of p53 alone on the promoters used in each assay.

Cell transformation assay

Rat Embryo Fibroblasts (REFs) obtained from Biowhittaker were grown in DMEM to 50% confluence in a 90mm dish. Cells were then transformed as described

previously [14]. Briefly, 2μg of EJ ras 6.6, 2μg of pCB (E1A), 5μg of pCB6-16E, 5μg of wild type human p53, 1μg or 5μg of human or C. elegans iASPP were transfected into the REFS as indicated. All the cells were transfected with the same amount of plasmid DNA expressing neo gene. The transfected cells were then selected with 400μg/ml of G418 and the morphologically transformed colonies were scored after 3-4 weeks after transfection.

Flow cytometry

For FACS analysis, 10^6 Saos-2 cells were plated 24-48 h prior to transfection in 10cm plates. The cells were then transfected with 2μg of a plasmid expressing CD20. CD20 expression was used as a transfection marker. The transfections consisted of 1μg of human p53 or 4μg of Ce-p53,10μg of ASPP1 and ASPP2, 2μg of Bax, 15μg of anti-sense iASPP, 7.5μg-10μg of human iASPP or 7.5μg of Ce-iASPP plasmid as indicated. 36 hours after the transfection, both attached and floating cells were harvested using 4 mM EDTA/PBS and stained with FITC conjugated anti-CD20 antibody CD20Leu. For each experiment, one dish of cells was transfected with the control vector only without CD20. These cells were later stained with antibody CD20Leu under the same conditions as those co-transfected with CD20 plasmid to serve as a negative control. The cells lacking expression of CD20 plasmid were used to set the base line to allow the gating of the CD20 positive (and hence transfected) cells. After staining with the antibody CD20Leu, the cells were fixed and stained with propidium iodide. The DNA content of all the cells expressing CD20 was analysed using the flow cytometer (Becton Dickson) as described[15].

Protein biochemistry

For western blotting, cells grown in monolayers were washed with 1X PBS and lysed in either NP40 lysis buffer (1% Nonidet P40, 50 mM Tris pH8.0, 150 mM NaCl, 1 mM EDTA pH 8.0) or luciferase reporter lysis buffer. The protein concentration of the cell extracts was determined against a standard curve using the BioRad protein

assay system (BioRad). Between 15-100 µg of extract was mixed with 5X sample buffer and loaded on SDS-PAGE gels. The gels were wet transferred on to Protran nitrocellulose membrane and the resulting blots blocked in 10% reconstituted milk powder in 1X PBS. Subsequently the blots were incubated with primary antibody prepared either in tissue culture medium or as undiluted hybridoma supernatant and incubated with the appropriate secondary HRP conjugated antibody (Dako). In between each stage the blot was washed with repeat changes of TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween 20). The blot was exposed to hyperfilm following the use of ECL substrate solution (Amersham Life Science).

For immunoprecipitation cells were lysed in NP40 lysis buffer on ice for 30 minutes and pre-cleared with protein G beads for 1 hour at 4 C. The protein concentration was determined and 1000µg of the extract was incubated with antibody pre-bound to protein G beads for 4 hours at 4 C. The beads were washed twice in NP40 lysis buffer and twice in NET buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0). The IP beads were mixed with 5X sample buffer and loaded onto a SDS-PAGE gel.

In vitro translation and in vitro immunoprecipitation

ASPP family members and p53 were in vitro translated and labelled with ^{35}S - Methionine using the TNT T7 Quick coupled Transcription/Translation System (Promega). For the experiments shown in figure 1E, 15, 30 and 45µl of in vitro translated lysates of iASPP were added in addition to p53 and ASPP2. Rabbit anti-p53 antibody CM1 was used to detect the presence of unlabelled p53.

For figure 6A, 5-10µl of human iASPP lysate (unlabelled) was incubated with 15 µl of the lysate containing the in vitro translated Ce-p53. The mixture of proteins was allowed to co-translate at 30 C for 1 hr. 200µl of phosphate-buffered saline (PBS) was then added to the mixture of proteins and incubated at 4 C for a further hour on a rotating wheel. The anti-V5 antibody immobilised on protein G agarose beads was

added to the binding reactions and incubated on a rotating wheel at 4 C for16 hours. The beads were then washed with PBS. The bound proteins were released in SDS gel sample buffer and analysed by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Ce-p53 was detected by autoradiography and Human iASPP was detected by anti-V5 antibody following a western blot. For the rest of the figures, the proteins were labelled with ^{35}S -Methionine and immunoprecipitated as above using the indicated antibodies. Results were visualised using autoradiography.

In vivo labelling of cells with ^{35}S -methionine and ^{35}S --cystiene

U20S cells in the absence or presence of transfected plasmids (as indicated in figure 1A and 2C) were washed with PBS and then incubated with 250µci/ml of ^{35}S - methionine and 250µci of 35-cysteine in DMEM lacking both methionine and cysteine for 2 hours at 37C. Cells were then washed with PBS before harvesting. For figure 2C, twenty four hours after transfection the cells were in vivo labelled with ^{35}S-methionine and ^{35}S-cysteine for 2hrs. Cells expressing CD20 (transfected cells) were stained with FITC-conjugated anti-CD20 antibody. A biotin conjugated anti-FITC antibody was then added to the cell pellet, and after the incubation, the cells were mixed with streptavidin conjugated magnetic beads to isolate the CD20 expressing cells. The cells were then lysed with NP40 lysis buffer and the proteins immunoprecipitated with mouse anti iASPP antibody. The immunoprecipitates were washed with NET buffer and resolved by SDS-PAGE and autoradiography.

Cell Fixation

Monolayers of cells were grown in 30 mm dishes and washed with 1X PBS. Cells were fixed with 1 ml of 4% para-formaldehyde for 15 minutes and then washed in 1X PBS. 1 ml of 0.2% Triton-X100 in 1X PBS was used to permeabilise the cells for 2 minutes and this was washed off with three washes of 1X PBS. Primary antibody was prepared in tissue culture medium at the appropriate concentration and added to the dishes for 3 hours. The dishes were washed with 1X PBS and the secondary

antibody of either anti-rabbit TRITC (Tetramethyl rhodamine isothiocyanate) or anti-mouse FITC (Fluorescein isothiocyanate) prepared in tissue culture medium at the manufacturers recommended dilution (Sigma, UK) and added to the dishes for one hour. The cells were washed in 1X PBS and left to air dry. Citifluor shielding agent (Citifluor, UK) was applied as a drop to the surface of the cells and a cover slip placed on top. A drop of immersion oil on top of the cover slip allowed the immunocomplexes to be visualised using a Zeiss Axiophot fluorescence microscope. Antibodies 9E10 and V5 were used to detect the expression of epitope tagged Ce-p53 (9E10), human iASPP (V5) and Ce-iASPP (V5) respectively. Human p53 was detected by DO.1 antibody.

Cloning Ce-p53 and Ce-iASPP cDNA

cDNAs carrying the complete coding regions of Ce-ape-1 (iASPP) and Ce-cep-1 (p53) were generated by RT-PCR using the Promega Access kit, cloned into the vector pCR4-TOPO (Invitrogen) and sequenced. C. elegans p53 and iASPP were then subcloned into a mammalian expression vector pcDNA3 in frame with the epitope of 9B10 and V5 respectively. The full-length Ce-ape-1 is predicted to be trans-spliced to SL1 (Y. Kohara, unpublished).

RNA interference (RNAi) and cell corpse assays

RNAi was performed by feeding or microinjection using established procedures (Fire et al., 1998; Timmons and Fire, 1998) In order to eliminate Ce-iASPP and Ce-p53 activity by RNAi, N2 animals were first subjected to Ce-iASPP RNAi feeding. Subsequently, 20 F1 animals were removed from the feeding plate, injected with Ce-p53 dsRNA and returned to independent Ce- iASPP dsRNA feeding plates. The F2 progeny of animals fed Ce-iASPP (+/- injection of Ce-p53 dsRNA) were stained with SYTO12 and apoptotic corpses scored as described12. Hence, because all animals have been subjected to Ce-iASSP feeding RNAi, any differences in the mean number

of apoptotic cell corpses detected in the two populations are likely to result from Ce-p53 dsRNA injection.

iASPP is the most conserved member of the ASPP family

Sequence analysis indicates that the C. elegans p53 gene, cep-1, is a distant member of the p53 family; however, the residues critical for ASPP and DNA binding activity appear to be conserved[3,4] Hence, we searched the C. elegans genome for an ASPP homologue and found that F46F3.4 is the only C. elegans gene encoding a protein with significant sequence homology to all three members of the ASPP family. The gene corresponding to F46F3.4 has been named ape-1 (for apoptotic enhancer) based on the mutant phenotype produced by ape-1(RNAi) (see below); however, the protein product will henceforth be referred to as Ce-iASPP. Ce-iASPP consists of 769 amino acids; sequence comparisons reveal that the C-terminus of Ce-iASPP is the region most conserved with other ASPP members (figure 7A). It was previously shown that the C-terminus of ASPP2 interacts with p53. Moreover, all but one (6 out of 7) residue involved in this interaction are conserved in both iASPP and Ce-iASPP. Taken together these results suggested that Ce-iASPP might interact with both human and C. elegans p53. This was tested in vitro by co-immunoprecipitation. As shown in figure 7B, Ce-iASPP interacts with both human and Ce-p53. The interaction between Ce-p53 and Ce-iASPP was further confirmed by reciprocal immunoprecipitation (Figure 7B, right panel).

Ce-iASPP contains the hallmark ankyrin repeats and SH3 domain found in other ASPP family members and is expressed in the cytoplasm and nucleus of human cells (figure 7C). The expression of human iASPP is predominantly nuclear but cytoplasmic staining is also detectable. Both human and C. elegans p53 are primarily expressed in the nucleus of transfected human cells (figure 7C). Since ASPP and iASPP can positively and negatively regulate the apoptotic function of p53, respectively, we tested the effect of Ce-iASPP on the activities of p53. When Ce-iASPP was co-expressed with human p53 in mammalian cells, it produced a small

reduction in the transactivation and apoptotic function of p53, presumably by inhibiting endogenous ASPP function. However, in the presence of ASPP1 or ASPP2, co-expression of Ce-iASPP prevented ASPP1 or ASPP2 from stimulating the transactivation and apoptotic function of p53 to the same extent as human iASPP (figure 7D). Furthermore, the ability of human and C. elegans iASPP to inhibit apoptosis is p53-dependent since they both failed to inhibit Bax induced apoptosis under the same conditions (figure 7E). The ability of iASPP to inhibit the activity of p53 is not due to the reduced expression of p53 (figure 7F). Like human iASPP, the Ce-iASPP also has oncogenic activity. The expression of Ce-iASPP enhanced the transforming activity of ras and E1A. Moreover, expression of Ce-iASPP inhibited the suppressor function of wild type human p53 (figure 7G). These results demonstrate that Ce-iASPP is more likely to be an orthologue of human iASPP than of ASPP. Also, the ability of Ce-iASPP to inhibit p53 suggests that the regulation of p53 apoptotic function by the ASPP family of proteins is evolutionarily conserved.

The regulation of p53 by ASPP family of proteins is evolutionarily conserved

It was unclear whether Ce-p53 could induce apoptosis in mammalian cells because of the limited sequence similarity between human and C. elegans p53, although most of the Ce-p53 residues that contact ASPP are conserved. If Ce-iASPP inhibits the activities of human p53 in a manner similar to that of human iASPP, this argues that the regulation of p53 by the ASPP family has been evolutionarily conserved. This further suggests that the activities of Ce-p53 could be subject to regulation by the ASPP family of proteins. To address these issues, Ce-p53 was tested for its ability to interact in vitro with members of the human ASPP family by co-immunoprecipitation. As shown in figure 6A, Ce-p53 interacts with ASPP2 and iASPP. The expression of Ce-p53 induced apoptosis in human cells with an efficiency similar to human p53. Remarkably, expression of human ASPP, ASPP2 in particular, significantly enhanced the ability of Ce-p53 to induce apoptosis to an extent similar to human p53 and the expression of human iASPP also inhibited the apoptotic function of Ce-p53 (figure 6B). In addition, both human and C.elegans

iASPP inhibited Ce-p53 induced apoptosis to the same extent (figure 6C). The ability of Ce-p53 to transactivate p53 target gene promoters, such as Bax-luc, was also tested and was found to be much lower than that of human p53. Interestingly, co-expression of ASPP2 and Ce-p53 resulted in a very small but detectable increase in the transactivation function of Ce-p53, indicating that the human ASPP family could regulate Ce-p53 in a manner similar to that of human p53 (figure 6D). The small increase in the transactivation function of Ce-p53 by ASPP2 is not observed on the mdm2 promoter. All of these results suggest that the residues conserved between human and C. elegans p53 are crucial and sufficient for the apoptotic function of p53 and for p53 to be regulated by the ASPP family.

iASPP is an evolutionarily conserved inhibitor of p53 in vivo

Like human p53, one of the most important functions of C. elegans p53 is its ability to induce apoptosis in germ cells in response to DNA damage [3,4]. Knowing that co-expression of human or C. elegans iASPP can inhibit the apoptotic function of p53 in mammalian cell lines, we hypothesised that expression of Ce-iASPP might similarly protect C. elegans germ cells from death by apoptosis. This question was addressed in vivo using RNA mediated interference (RNAi) [5]. Depletion of endogenous Ce-iASPP increased the number of germ cells undergoing apoptosis, indicating that the normal function of Ce-iASPP is to inhibit apoptosis (figure 8A, lanes 1 and 5, 6). The enhancement of germ cell apoptosis caused by depletion of Ce-iASPP was not detected when RNAi was performed in a mutant lacking the C. elegans CED-3 caspase, indicating that the core apoptotic machinery is involved in this process (figure 8A, lanes 3 and 4) [6]. We also obtained additional support for the hypothesis that the primary role of Ce-iASPP is to inhibit the pro-apoptotic activity of Ce-p53, which is normally stimulated in response to genotoxic stress [3,4]. First, it was found that the increase in the number of C. elegans germ cells undergoing apoptosis after depletion of Ce-iASPP was abrogated by simultaneously depleting Ce-p53 by RNAi (figure 8A, compare lanes 5, 6 and 8). Moreover, the depletion of both Ce-iASPP and Ce-p53 by RNAi did not completely eliminate apoptosis, but instead returned the

number of germ cells undergoing apoptosis to wild-type physiological levels. Second, the increase in the number of apoptotic germ cell corpses detected after wild type worms were exposed to 100-Gy IR was no greater than that observed after depletion of Ce-iASSP by RNAi in the presence or absence of exposure to 100-Gy IR (figure 8A, lanes 2 and 7). These results clearly demonstrate that iASPP is an important inhibitor of p53 function in C. elegans, although we can not exclude the possibility that a genetic knockout might reveal that Ce-iASPP has additional activities. Since the regulation of p53 by the ASPP family is highly conserved, it is likely that iASPP is also a key inhibitor of p53 in other organisms including humans.

We show here that iASPP is the most phylogenetically conserved inhibitor of p53, so far identified, and also the most evolutionarily conserved member of the ASPP family. Remarkably, the ability of ASPP family members to regulate the apoptotic function of p53 has been conserved between C. elegans and human. This argues that the apoptotic function of p53 is likely to be more conserved than its ability to induce cell cycle arrest; this agrees with recent observations showing that ectopic expression of both C. elegans and Drosophila p53 induces apoptosis but not cell cycle arrest [3,4,7,8]. In C. elegans p53-mediated apoptosis appears to play an important role in maintaining the fidelity of germ cells, which might have incurred DNA damage [3,4]. Interestingly, the most important tumour suppressor function of p53 is also linked to its ability to induce apoptosis. Therefore, ASPP family members, the evolutionarily conserved regulators of p53, must play a critical role in tumourigenesis.

The oncoprotein iASPP

iASPP shares more sequence similarity with the N-terminally truncated ASPP2 mutant 53BP2 than the full-length ASPP. Expression of iASPP inhibited the apoptotic function of p53. Like 53BP2, the most profound effect of iASPP on the apoptotic function of p53 is mediated through its ability to act as a competitor of ASPP. However, in C. elegans, iASPP is the only gene that has homology to the human ASPP family. Thus iASPP directly inhibits the apoptotic function of p53 in C.

elegans. A similar mechanism might also apply in mammalian cells. In agreement with this, iASPP antisense RNA induced a 3 to 5-fold increase in apoptotic cells in U2OS and MCF7 cells. In this latter model, ASPP could stimulate the apoptotic function of p53 by removing the negative effects that iASPP imposed on p53. The failure of iASPP antisense RNA to produce a significant increase of apoptosis in cisplatin treated U2OS and MCF7 cells might be due to the fact that cisplatin stimulates the apoptotic function of p53 by increasing the activities of ASPP. This, in turn, might explain why the expression of ASPP antisense RNA generated a profound inhibitory effect on apoptosis induced by cisplatin. It is also under this condition that the anti-apoptotic function of iASPP is most pronounced. Thus the apoptotic function of p53 is negatively regulated by iASPP and positively regulated by ASPP. The competition between these two opposing signals could determine the apoptotic state of p53 and ultimately cell fate. Regardless of whether iASPP acts as a dominant negative regulator of ASPP or a direct inhibitor of p53, a competition between iASPP and ASPP for binding to p53 is critical for the apoptotic function of p53. Consistent with this model, a change in the percentage of p53 complexed with ASPP2 was seen in response to DNA damage. It is likely that the percentage of p53 complexed with iASPP and ASPP would be regulated by signals that induce death or survival.

Being an inhibitor of p53, iASPP enhanced the transforming activities of oncogenes such as ras plus E7 or E1A of human papilloma virus and adenovirus but not ras plus mutant p53. This is particularly interesting since E7 and E1A are known to induce p53-dependent apoptosis [9]. While E7 and E1A can bind and inactivate the tumour suppressor function of Rb, their oncogenic function is largely reduced due to their ability to activate p53-dependent apoptosis. Proteins that can inhibit apoptosis induced by E7 and E1A would enhance the oncogenic function of E7 and E1A. Therefore, like the dominant negative p53 mutants, iASPP was able to stimulate the oncogenic function of E7 and E1A by inhibiting the apoptotic function of p53. It is important to point out that iASPP was not as active as mutant p53, p53H175 or p53L173 in co-operating with ras to transform REFs under the experimental

conditions described here. Part of the reason was due to the low expression level of iASPP in the assay (data not shown). However, the differences in the transforming activities of iASPP and mutant p53 might also be caused by other known activities of mutant p53, which are independent of its ability to act as a simple dominant negative inhibitor of p53. Nevertheless the ability to confer cellular resistance to the cytotoxic effects of UV and cisplatin suggested that the overexpression of iASPP would be selected for in human tumours expressing wild type p53. Consistent with this, iASPP expression is increased in human breast carcinomas expressing wild type p53. The majority of tumours (7 out of 8) expressing high levels of iASPP also express wild type p53 and normal levels of ASPP, indicating that iASPP is an inhibitor of ASPP in vivo. Our previous study showed that the expression levels of ASPP1 and ASPP2 were down regulated in 60% of human breast tumours express wild type p53. Taken together, the abnormal expression of ASPP family members would account for almost 80% of human breast carcinomas examined. The ASPP family members are encoded by three different genes located on different chromosomes (data not shown). We do not know why the frequency of human breast carcinomas showing down regulation of ASPP is much higher than those showing increased expression of iASPP. However, it is possible that the expression pattern of the ASPP family members varies in different types of human tumours. The percentage of tumours with altered ASPP expression could also differ in various tumour types. Nevertheless, inhibiting the oncogenic function of iASPP could provide an important new strategy to treat tumours expressing wild type p53.

Evolutionarily conserved regulation of p53 by the ASPP family

Sequence comparison reveals that there is 38% identity between the human and C. elegans iASPP amino acid sequences; within the ankyrin repeats and SH3 domain, the homology is as high as 78% (residues 154-227 of human iASPP and 557-630 of Ce-iASPP, 55/74 residues are similar). Most of the iASPP residues contacting p53 are conserved. The structural conservation between human and C. elegans iASPP is reflected by their ability to regulate p53 function in human cells. Like human iASPP,

C. elegans iASPP interacts with and inhibits the transactivation and apoptotic function of human p53 in cell lines. The conservation of ASPP/p53 regulation is further demonstrated in a study of C. elegans p53 in human cells. It is interesting and important to point out that the sequence homology between human and C. elegans p53 is very limited (13.7% identity at protein level). The highest level of p53 homology between the two species is around 50% in a very limited region (residues 9/18 residues are similar). However, many of the ASPP2 contact residues identified from a crystal structure[10] are conserved between human and C. elegans p53 (5 out of 8 residues are conserved). The ability of C. elegans p53 to interact with human ASPP family members in vitro highlights the importance of these conserved residues. Remarkably, C. elegans p53 induces apoptosis very effectively in human cells. Similar to human p53, the apoptotic function of C. elegans p53 is positively and negatively regulated by the human ASPP and iASPP, respectively. These results demonstrate for the first time that the apoptotic function of p53 is conserved despite the limited sequence homology between human and C. elegans p53. The few key residues conserved between human and C. elegans p53 are sufficient for ASPP family members to regulate the apoptotic function of p53 both in vitro and in vivo.

The C. elegans p53 showed little ability to transactivate human p53 target genes such as Bax and PIG3 in comparison to human p53 (data not shown). Even though Bax is frequently a p53 target gene during apoptosis, it is not the only target gene that is required for p53 induced apoptosis. p53 is known to transactivate over 20 pro-apoptotic genes none of which has so far proved to be indispensable in p53 induced apoptosis. There are over 4000 putative p53 target genes in the human genome and many of them are pro-apoptotic genes [11]. It is possible that Ce-p53 might transactivate some of these other human p53 target genes as effectively as human p53. Unfortunately none of the ones we tested so far belong to this category. Although transactivation of the human Bax-luc reporter in human cells by Ce-p53 is not very strong, it is important to point out that the co-expression of ASPP stimulated the transactivation function of Ce-p53 only on the promoters of Bax but not on mdm2. This pattern of ASPP action is similar to that seen with human p53.

Alternatively, Ce-p53 might induce apoptosis independent of its transcriptional activity; human p53 is known to induce apoptosis through both transcriptional dependent and independent pathways [12]. Regardless of how Ce-p53 induces apoptosis in human cells, the most remarkable and important fact is that the human ASPP family of proteins regulate the apoptotic function of both Ce-p53 and human p53 in a similar way. Ce-iASPP is also able to completely replace human iASPP in all the assays performed in human cells. These results argue strongly that the regulation of p53 function by ASPP family is conserved from worm to human. The link between p53 and the ASPP family suggests that the regulation of p53 by the ASPP family should be the future target in developing new strategies for cancer therapy. Tumours expressing wild type p53 can be sensitised to treatments by enhancing the activity of ASPP and removing the activity of iASPP.

Claims

1. An isolated nucleic acid molecule which encodes a polypeptide, or sequence variant thereof, wherein said polypeptide is a fragment of the polypeptide sequence represented in Figure 1a or 1b, which fragment is selected from the group consisting of:

i) a polypeptide fragment consisting of amino acid residues from about residue 128-224 of the amino acid sequence presented in Figure 1a or 1b;

ii) a polypeptide fragment consisting of amino acid residues from about residue 128-224 of the amino acid sequence presented in Figure 1a or 1b wherein said sequence has been modified by addition, deletion or substitution of at least one amino acid residue; and

iii) a polypeptide as defined in (i) and (ii) wherein said polypeptide substantially retains the biological activity of the polypeptide represented in Figure 1a or 1b.

2. A nucleic acid molecule according to Claim 1 wherein said molecule encodes a fragment consisting of amino acid residues from about residue 128-224 of the sequence represented in Figure 1a.

3. A nucleic acid molecule according to Claim 2 wherein said molecule is isolated from a human.

4. A nucleic acid molecule according to Claim 1 or 2 wherein said molecule encodes a fragment consisting of amino acid residues from about residue 128-224 of the sequence represented in Figure 1b.

5. A nucleic acid molecule according to Claim 4 wherein said molecule is

isolated from a nematode.

6. A nucleic acid molecule according to Claim 5 wherein said nematode is of the genus *Caenorhabditis spp.*

7. A nucleic acid molecule according to any of Claims 1-6 wherein molecule encodes a polypeptide, or sequence variant thereof, which polypeptide inhibits the activity of a polypeptide represented by the amino acid sequence represented in Figure 2.

8. A nucleic acid molecule according to any of Claims 1-7 wherein said nucleic acid molecule is a cDNA.

9. A nucleic acid molecule according to any of Claims 1-7 wherein said nucleic acid molecule is genomic DNA.

10. A polypeptide fragment or sequence variant thereof, encoded by a nucleic acid molecule according to any of Claims 1-9.

11. A vector comprising a nucleic acid according to any of Claims 1-9.

12. A vector according to Claim 11 wherein said vector is an expression vector.

13. A cell tranformed or transfected with a nucleic acid molecule according to any of Claims 1-9 or vector according to Claim 11 or 12.

14. A nucleic acid according to any of Claims 1-9 for use as a pharmaceutical.

15. A polypeptide according to Claim 10 for use as a pharmaceutical.

16. A nucleic acid or polypeptide according to Claim 14 or 15 further comprises a a diluent, carrier or excipient.

17. A transgenic non-human animal comprising a nucleic acid according to any of Claims 1-9.

18. The use of the polypeptide, or fragment thereof, according to Claim 10 in a screening method for the identification of agents which inhibit the binding of said polypeptide to p53.

19. A screening method to identify agents which inhibit the binding of a polypeptide or fragment thereof to p53 comprising:

i) forming a preparation comprising
   c) a polypeptide according to the invention; and
   d) a p53 polypeptide, or a fragment thereof consisting of the binding site(s) for the polypeptide in (a);

ii) providing at least one agent to be tested; and

iii) determining the activity of the agent with respect to the binding of the polypeptide in (a) to the polypeptide in (b).

20. A method according to Claim 19 wherein said agent is a polypeptide.

21. A method according to Claim 20 wherein said polypeptide is a peptide.

22. A method according to Claim 20 wherein said polypeptide is an antibody or binding part thereof.

23. A method according to Claim 22 wherein said antibody is a monoclonal antibody.

24. A method according to Claim 22 or 23 wherein said fragment is a Fab fragment.

25. A method according to Claim 24 wherein said Fab fragment is selected from the group consisting of: $F(ab')_2$, Fab, Fv and Fd fragments; and CDR3 regions.

26. A method according to any of Claims 23-25 wherein said said antibody is a humanised.

27. A method according to any of Claims 23-25 wherein said antibody is a chimeric antibody.

28. An isolated nucleic acid molecule wherein said molecule is isolated from a nematode worm which nucleic acid molecule hybridises a nucleic acid sequence as represented by Fig 1b, wherein said nucleic acid molecule encodes an inhibitor of p53.

29 A nucleic acid molecule according to Claim 28 wherein said molecule hybridises under stringent hybridisation conditions.

30. A nucleic acid molecule according to Claim 28 or 29 wherein said nematode worm is of the genus *Caenorhabditis spp.*

31. An isolated polypeptide comprising the amino acid as represented in Figure 2b which polyeptide is modified by addition, deletion or substitution of at least one amino acid residue and is an inhibitor of p53.

32. A method of treatment of an animal comprising administering an effective amount of a polypeptide according to Claim 10 wherein said effective amount induces the apopoptic activity of p53.

33. A method of treatment of an animal comprising administering an effective

amount of a nucleic acid molecule according to any of Claims 1-9 or a vector according to Claim 11 or 12 wherein said effective amount induces the apopoptic activity of p53.

34. A method according to Claim 32 or 33 wherein said treatment is of cancer.

35. A peptide comprising an amino acid sequence selected from the group consisting of: DGPEETD; TTLSDG; AEFGDE; or PRNYFG.

36. A peptide according to Claim 35 wherein the length of said peptide is at least 6 amino acid residues.

37. A peptide according to Claim 35 wherein the length of said peptide is selected from the group consisting of: is at least 7 amino acid residues; 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues.

38. A peptide according to Claim 35 wherein the length of said peptide is at least 20 amino acid residues; 30; 40; 50; 60; 70; 80; 90; or 100 amino acid residues.

39. A peptide according to Claim 35 consisting of an amino acid sequence selected from the group consisting of: DGPEETD; TTLSDG; AEFGDE; or PRNYFG.

40. Use of a peptide selected from the group consisting of; DGPEETD; TTLSDG; AEFGDE; or PRNYFG as a pharmaceutical.

41. A pharmaceutical composition comprising a peptide selected from the group consisting of: DGPEETD; TTLSDG; AEFGDE; or PRNYFG.

42. A pharmaceutical composition according to Claim 41 wherein said composition further includes a carrier, diluent or excipient.

43. A method of treatment of an animal, preferably a human, wherein said animal would benefit from the induction of apoptosis comprising administering an effective amount of a peptide according to any of Claims 35-39.

44. A method according to Claim 43 wherein said treatment is cancer treatment.

ABSTRACT

POLYPEPTIDE

The invention relates to a polypeptide, or part thereof, which inhibits the apoptotic activity of the tumour suppressor protein p53 and including screening methods to identify agents which interfere with the activity of said polypeptide.

Table 1

IASPP cross-species homologies

	Human	Mouse	C.El.	Drosophila	Fugu I	Fugu II	Fugu III	Fugu IV
Human (352)	X	X	X	X	X	X	X	X
Mouse (260)	93.7	X	X	X	X	X	X	X
C.El. (769)	20.4	38.8	X	X	X	X	X	X
Drosophila (1071)	43.2	40.0	32.4	X	X	X	X	X
Fugu I (260)	51.9	45.0	46.1	55.4	X	X	X	X
Fugu II (252)	54.8	54.8	51.6	58.7	X	X	X	X
Fugu III (144)	54.2	53.5	54.2	64.6	X	X	X	X
Fugu IV (132)	51.5	50.8	55.3	62.9	X	X	X	X

Figure 1a

```
   1 GCGGCCGCGT CGACCCGGCG TTCAGACGCG GGCAGCTACC GGCGCTCGCT GGGCTCCGCG
  61 GGGCCGTCGG GCACTTTGCC TCGCAGCTGG CAGCCCGTCA GCCGCATCCC CATGCCCCCC
 121 TCCAGCCCCC AGCCCCGCGG GGCCCCGCGC CAGCGTCCCA TCCCCCTCAG CATGATCTTC
 181 AAGCTGCAGA ACGCCTTCTG GCAGCACGGG GCCAGCCGCG CCATGCTCCC TGGGTCCCCC
 241 CTCTTCACCC GAGCACCCCC GCCTAAGCTG CAGCCCCAAC CACAACCACA GCCCCAGCCA
 301 CAATCACAAC CACAGCCCCA GCTGCCCCAA CAGCCCCAGA CCCAACCCCA AACCCCTACC
 361 CCAGCCTCCC ACATCCGCAT CCCCAACAGA CATGGCCCCC TGTGAACGAA GGACCCCCCA
 421 AACCCCCCAC CGAGCTGGAG CCTGAGCCGG AGATAGAGGG GCTGCTGACA CCAGTGCTGG
 481 AGGCTGGCGA TGTGGATGAA GGACCCTGTA GCAAGGCCTC TCAGCCCCAC GAGGCTGCAG
 541 CCAGCACTGC CACCGGAGGC ACAGTCGGTG CCCGAGCTGG AGGAGGTGGC ACGGGTGTTG
 601 GCGGAAATTC CCCGGCCCCT CAAACGCAGG GGCTCCATGG AGCAGGCCCC TGCTGTGGCC
 661 CTGCCCCCTA CCCACAAGAA ACAGTACCAG CAGATCATCA GCCGCCTCTT CCATCGTCAT
 721 GGGGGGCCAG GGCCCGGGGG GCGGAGCCAG AGCTGTCCCC CATCACTGAG GGATCTGAGG
 781 CCAGGGCAGG GCCCCCTGCT CCTGCCCCAC CAGCTCCCAT TCCACCGCCC GGCCCCGTCC
 841 CAGAGCAGCC CACCAGAGCA GCCGCAGAGC ATGGAGATGC GCTCTGTGCT GCGGAAGGCG
 901 GGCTCCCCGC GCAAGGCCCG CCGCCGCGCC CTCAACCCTC TGGTGCTCCT CCTGGACGCG
 961 GCGCTGACCG GGGAGCTGGA GGTGGTGCAG CAGGCGGTGA AGGAGATGAA CGACCCGAGC
1021 CAGCCCAACG AGGAGGGCAT CACTGCCTTG CACAACGCCA TCTGCGGCGC CAACTACTCT
1081 ATCGTGGATT TCCTCATCAC CGCGGGTGCC AATGTCAACT CCCCCGACAG CCACGGCTGG
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1201 CACGGCGCTG CAATCTTCGC CACCACGCTC AGCGACGGCG CCACCGCCTT CGAGAAGTGC
1261 GACCCTTACC GCGAGGGTTA TGCTGACTGC GCCACCTACC TGGCAGACGT CGAGCAGAGT
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1381 GACGAGCTGT CCTTCCGCGA GGGCGAGTCG GTCACCGTGC TGCGGAGGGA CGGGCCGGAG
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1621 TTGCTGCCTT TATCTGCACC CCTCACCCTG CTGGTGGTGG TCCTTGCCAC CGGTTCTCTG
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1921 AGTCACTGCT GACACCATCT CTCCCAGCAG TCTTGGGGTC TGGGTGGGAA ACATTGGTCT
1981 CTACCAGGAT CCCTGCCCCA CCTCTCCCCA ATTAAGTGCC TTCACACAGC ACTGGTTTAA
2041 TGTTTATAAA CAAAATAGAG AAACTGGTTT AATGTTTATA AACAAAATAG AGAAACTTTC
2101 GCTTATAAAT AAAAGTAGTT TGCACAGAAA TGAAAAAAAA AAAAAAAAAA AAAAAA
```

Figure 1b

```
   1 atggtcacga ccagtagcgg agggggtata gggtacccgg caaacaacgg tgtcacacag
  61 gtgtctctga ttcactcgtc ggattctgta cgaactgttt caactgcccc aatataccgt
 121 ccgacgtcat caatggcatc tacgatggct cataaatctt cgacggctcc gttcatctcc
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 241 ccacaagcat tgtcccaaca gtatcaccag cagaatccga tgatgatgta ttccgcacca
 301 aatacacgac cacacgttat tccgacaatg caagtgcaac cgacaatggc cgctcaaatt
 361 aaacgaaata atcctgttaa tgcacagttt cagaaccctt ctgaaatgat cgccgattac

 421 ggtgtaaaac cgcagtcagt agaaatggtg caaagagttc gagctgttcg aagacaagtc
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 601 gctcaattag agttgagaaa tgcttcaatg agggcgcaat ctttaaacaa gcatttggaa
 661 gaaatgtacc ggagaagaca aactgcagca gcggcagcgc tcgtggaaca acgaaaaatg
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 841 cctcaaatga tgaaatcaga agaattttcg gagaaaagag atttgaatgg acaaactggc
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 961 ttggcaccat gtaaagaaaa ccagcaaaaa tactcggagt taagtaaaat ggcatctacg
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1201 ggaaagactg atttggtatc acttcgatca gattccctga aagctacgaa acgtcgttct
1261 tgggctgctt ccgaaggtac ttcaatgtca gaggcagaga tgattcatag gcttcttgat
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1381 tcggcaataa catcagaaac atatgccgaa gaagttgtca attcagaatc gaaacaagtt
1441 gctacaagtt cggattccac taataatctt gaattgccaa ccgaacaaat ggtattaggt
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2281 ccattggaat cgaacaacaa tgtcgaataa
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Figure 2a

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LLLPHQLPFHRPAPSQSSPPEQPQSMEMRSVLRKAGSPRKARRARLNPLVLLLDAALT

GELEVVQQAVKEMNDPSQPNEEGITALHNAICGANYSIVDFLITAGANVNSPDSHGWT

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Figure 2b

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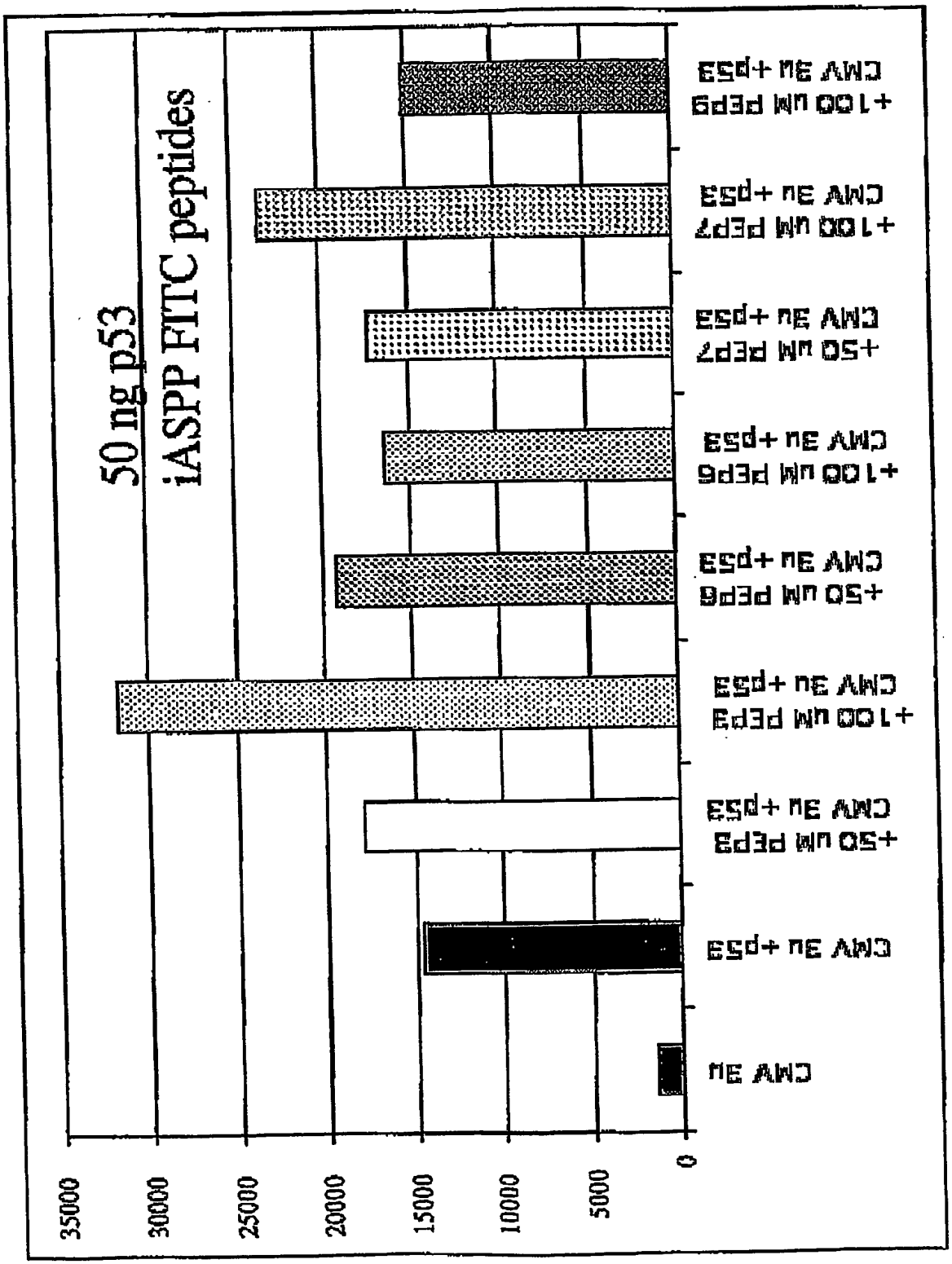
Figure 3a

Figure 3b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50 ng p53
iASPP FITC peptides
35000
30000
25000
20000
15000
10000
5000
0
CMV 3u
CMV 3u +p53
+50 uM PEP3
CMV 3u +p53
+100 uM PEP3
CMV 3u +p53
+50 uM PEP6
CMV 3u +p53
+100 uM PEP6
CMV 3u +p53
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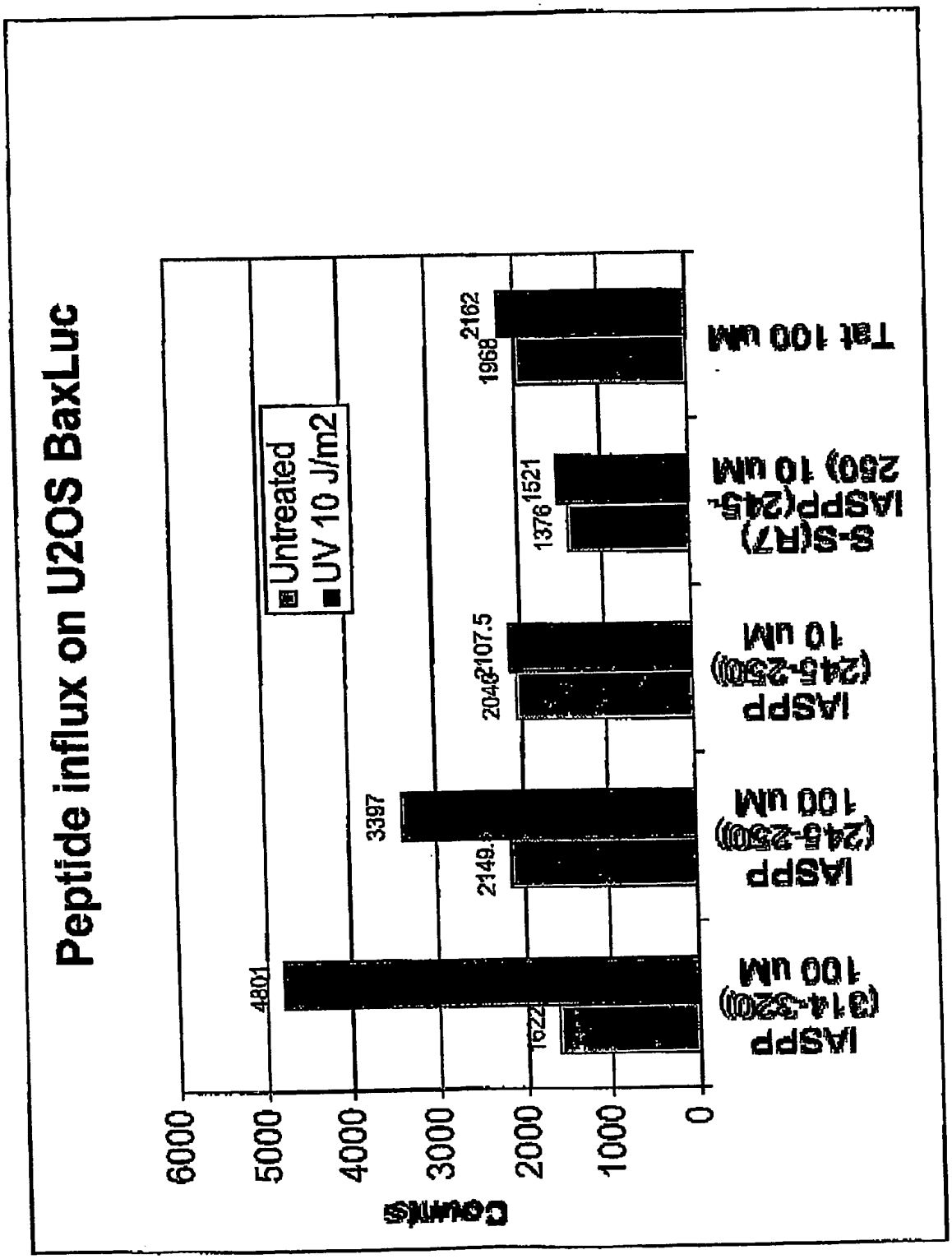
+100 uM PEP9
CMV 3u +p53

Figure 4

Figure 5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UV 10 J/m2
Counts
6000
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4000
3000
2000
1000
0
4801
1622
3397
2149
2042
2107.5
1376
1521
1968
2162
IASPP (314-320) 100 uM
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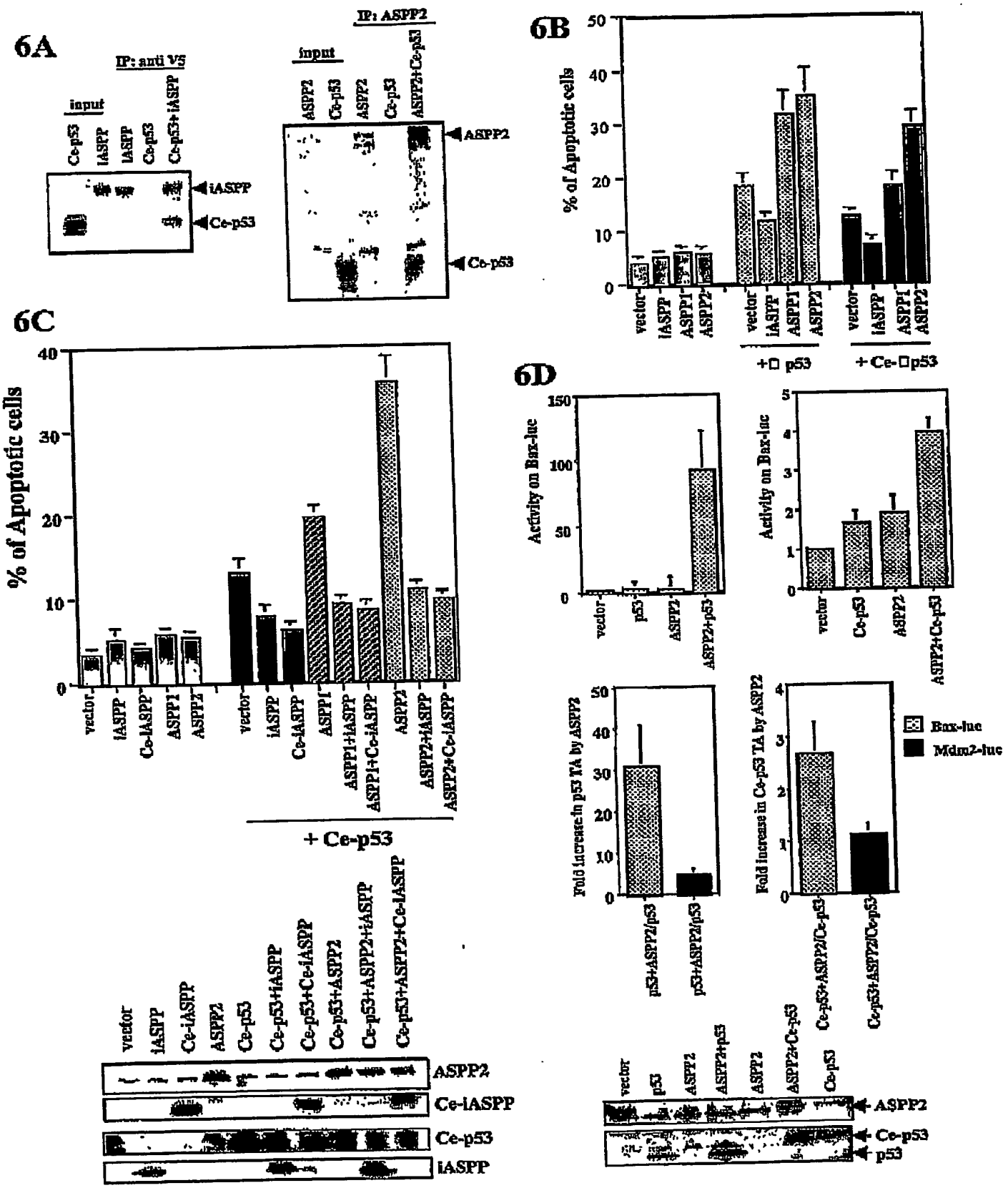
Tat 100 uM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Ce-p53
iASPP
iASPP
Ce-p53
Ce-p53+ iASPP
iASPP
Ce-p53
IP: ASPP2
input
ASPP2
Ce-p53
ASPP2
Ce-p53
ASPP2+Ce-p53
ASPP2
Ce-p53
6B
% of Apoptotic cells
vector
iASPP
ASPP1
ASPP2
+□ p53
+Ce-□p53
6C
% of Apoptotic cells
vector
iASPP
Ce-iASPP
ASPP1
ASPP2
ASPP1+iASPP
ASPP1+Ce-iASPP
ASPP2+iASPP
ASPP2+Ce-iASPP
+ Ce-p53
6D
Activity on Bax-luc
vector
p53
ASPP2
ASPP2+p53
Activity on Bax-luc
vector
Ce-p53
ASPP2
ASPP2+Ce-p53
Fold increase in p53 TA by ASPP2
p53+ASPP2/p53
Fold increase in Ce-p53 TA by ASPP2
Ce-p53+ASPP2/Ce-p53
Bax-luc
Mdm2-luc
vector
iASPP
Ce-iASPP
ASPP2
Ce-p53
Ce-p53+iASPP
Ce-p53+Ce-iASPP
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Ce-p53
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p53

7A

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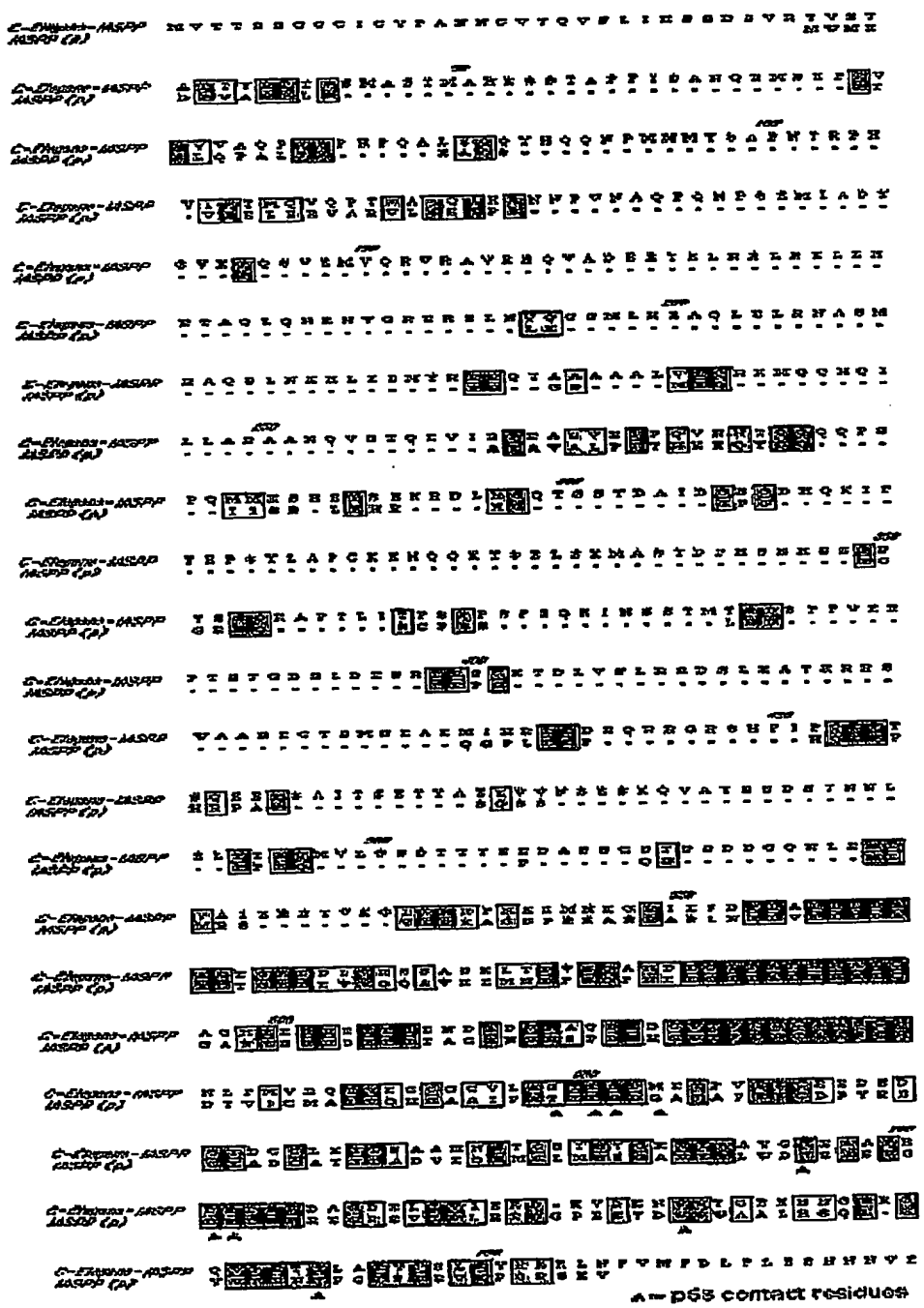

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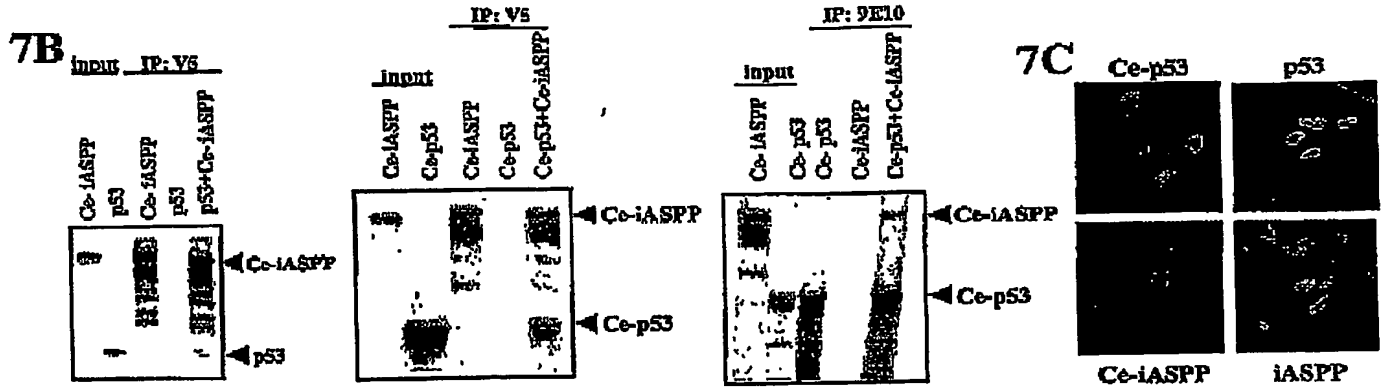
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p53
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Ce-iASPP
p53
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Ce-p53
Ce-iASPP
Ce-p53
Ce-p53+Ce-iASPP
Ce-iASPP
Ce-p53
Input
IP: 9E10
Ce-iASPP
Ce-p53
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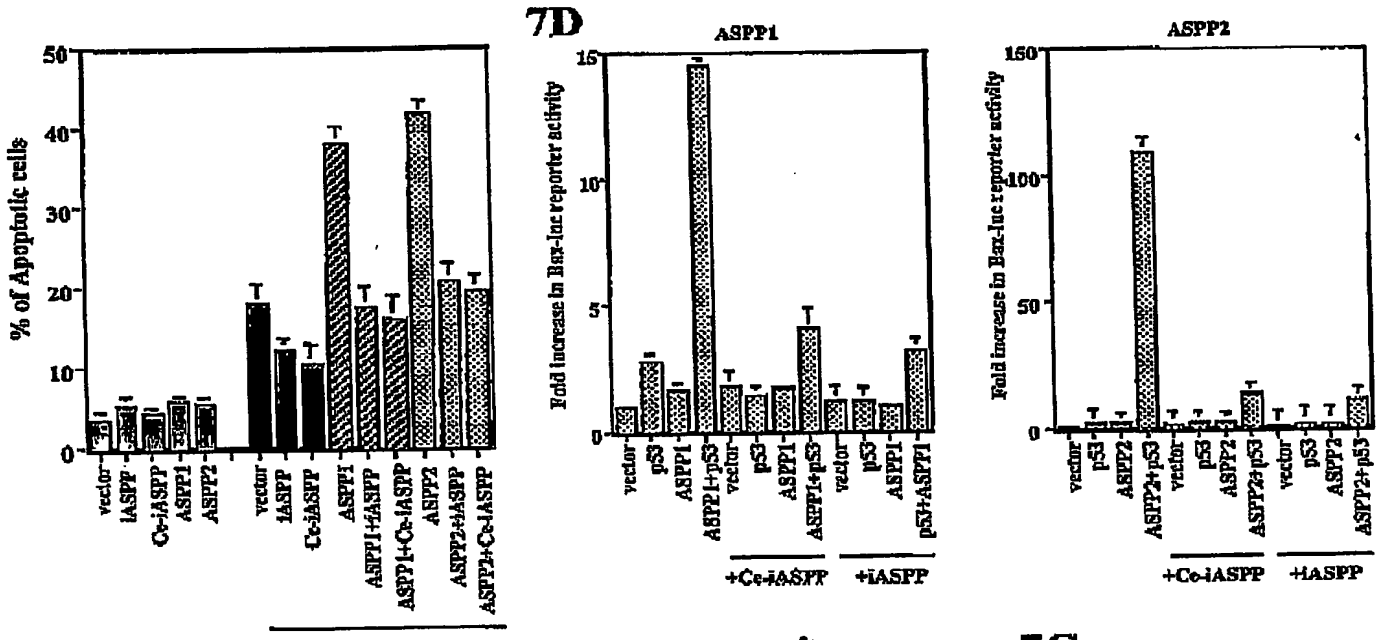
iASPP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ASPP1+iASPP
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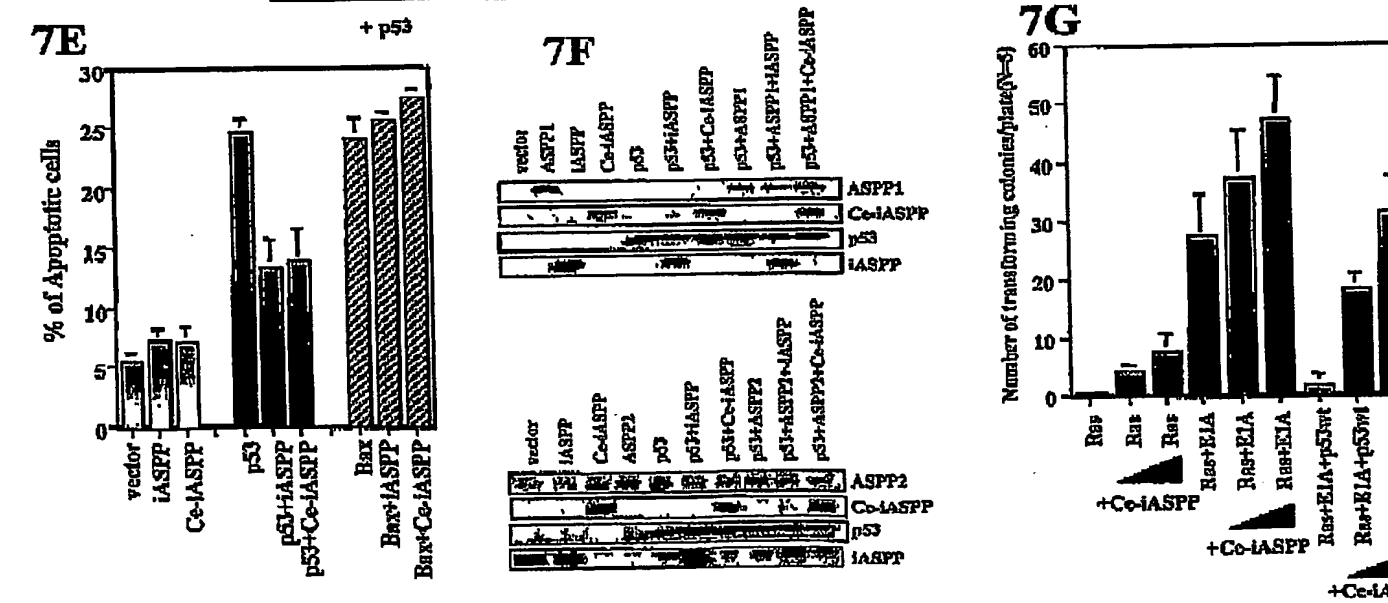
ASPP2+iASPP
ASPP2+Ce-iASPP
+ p53
7D
ASPP1
Fold increase in Bax-luc reporter activity
ASPP1+p53
+Ce-iASPP
+iASPP
ASPP2
ASPP2+p53


7E
% of Apoptotic cells
vector
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Bax
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Bax+Ce-iASPP
7F
ASPP1
Ce-iASPP
p53
iASPP
ASPP2
Ce-iASPP
p53
iASPP
7G
Number of transforming colonies/plate(N=6)
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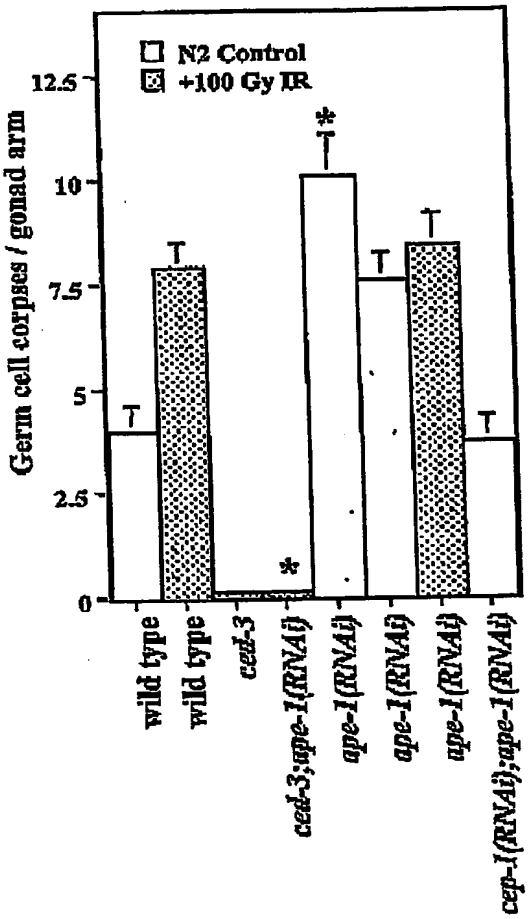
Ras+EIA
Ras+EIA+p53wt
+Ce-iASPP

ape-1 = iASPP
cep-1 = *Ce*-iASPP

8A



8B